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Studies of enzymes in milk produced with normal feed and protein-free feed

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Cow's milk contains a number of enzymes. Obviously they are products formed by the mammary gland and transferred to milk during the secretory process. At present it is known that there are at least 19 enzymes in milk. Although there are substrates for most of these enzymes, very little is known about the significance of these enzymes in milk. In dairy technology some of these enzymes are of considerable importance.

Since the milk enzymes form a small part of the total milk proteins, we have included also measurements of the activities of some of the main enzymes, namely aldolase, amylase, alkaline phosphatase, catalase, peroxidase and xanthine oxidase, in our protein studies (VIRTANEN and SYVÄOJA, 1964, SYVÄOJA and VIRTANEN, 1965) in order to find at the same time whether the purified protein-free test feed containing urea and small amounts of ammonium salts as the sole nitrogen source (VIRTANEN, 1966), has had any influence on the enzyme activities. Experiments have been performed only for a short time, so that nothing is known about the fluctuations with lactation period. Since the feed of the test cows remains qualitatively the same throughout the year no changes caused by the feeding are to be expected.

Aldolase

Aldolase is important in the metabolism of carbohydrates. It catalyzes the hydrolysis of fructose-1,6-diphosphate to dihydroxyacetone phosphate and phosphoglyceric aldehyde. Aldolase is found in milk in about the same concentration as in the blood and it is very unstable. The enzyme is associated with the fat globules and is concentrated in the cream layer when the milk is separated (POLIS and SHMUKLER, 1950). It has not yet been purified sufficiently for characterization of its properties and origin.

The milk aldolase activity was determined according to the „Mercotest“ aldose method. The triosephosphates formed from hexosephosphate by the aldolase were stabilized with hydrazine and the reaction was stopped with trichloracetic acid. In alkaline solution the trioses react with 2,4-dinitrophenylhydrazine to form colored hydrazones. The intensity of the color is proportional to the amount of fructose-1,6-diphosphate split per unit of time and thus also to the activity of the aldolase. The aldolase activities are usualy given in terms of the international unit (IU) which means that amount of enzyme per volume of 1000 ml that splits one micromole of substrate at 37 ° C in one minute. One milli-unit is one thousandth of international unit. The results are given in milli-units/ml in Table 1.

Table 1

Cow	aldolase activity in milli-unit/ml (37°C)
Aino } protein-free test feed	2.14
Jairu } protein-free test feed	1.31
Lila, potato, sugar beet pulp, and urea feed	1.22
Control, mixed milk on normal feed from the farm	2.20

Amylase

Amylases hydrolyze α-1,4-D-glucosidic bonds of starch and glycogen. α-Amylases cause a rapid decrease in the viscosity of starch solutions (= the liquefying and dextrinizing enzyme), free sugar being formed slowly. The β-amylases identified in plants function as exo-glycosidases (the saccharifying enzyme).

Several different scientists have observed α-amylase activity in milk. Richardson and Hankinson (1936) gave evidence that it is a native protein of milk. They also suggested that milk should have weak β-amylase activity. There is, however, no evidence that this would be a normal component of milk.

The concentration of α-amylase in milk is small and the enzyme is very labile, being especially sensitive to heat. α-Amylase is concentrated in the skimmed milk after separation and in whey after precipitation of casein with acid, although a part of it remains with casein. When serum proteins are fractionated, α-amylase has been shown to associate to a great extent with the lactoglobulin fraction.

This fraction has been used as the starting material for concentrating and purifying the enzyme (Guy and Jenness, 1958). The enzyme has not yet been crystallized and, concerning its activity in milk, it is known only that it varies from cow to cow and is high in milk from mastitic udders. The significance of the enzyme is also as yet unexplained. It possibly plays some rôle in the metabolism of the little-studied glycoprotein of the mammary gland.

The enzyme activities of milk samples were determined by measuring the decrease in the amount of starch during incubation (Guy and Jenness, 1958; Bergmeyer, 1962). The optimum pH of milk amylase is 7.4 and for activation both Ca^{++} and Cl^{-} are required (Schloemer et. al. 1939). Its optimum temperature is 44 ° C but it is inactivated rapidly at this temperature. Determinations were made at 35 ° C.

The starch was decomposed by the α-amylase of milk to dextrin and sugar. Iodine colors the starch blue, but not the decomposition product. The intensity of the color is directly proportional to the concentration of starch in the sample, and the decrease in intensity to the amount of substrate decomposed, and thus the amylase concentration can be calculated (Richterich and Colombo, 1962). The α-amylase activities can be seen in Table 2.

Table 2

Cow		amylase activity IU (μMol/min/1000 ml, 37 ° C)
Aino	protein-free test feed	499
Jairu	protein-free test feed	1263
Lila		555
Control		1029

Alkaline phosphatase

The phosphatases catalyze the hydrolysis of phosphoric acid esters. According to their effective pH-optima they are denoted alkaline or acid phosphatases. In milk, alkaline phosphatase is a native component (GRAHAM and KAY, 1933). It has not yet been crystallized, but it has been isolated in a very pure form, and electrophoretic studies have shown it to be homogenous, possessing only phosphomonoesterase activity (MORTON, 1953). SHAHANI (1966) has reviewed evidence that there may be more than one alkaline phosphatase in milk.

The activity of alkaline phosphatase in milk fluctuates to a great extent. HAAB and SMITH (1956) observed that the milk phosphatase activity is correlated with milk yield, reaching a minimum 1—2 weeks after calving an then rising gradually to a maximum in 25 weeks. It is not apparently related to the breed of the cow or its feed. 30—40 % of the alkaline phosphatase is associated with the fat globules of cream, the remainder being found in skimmed milk, possibly as a part of the protein (MORTON, 1954).

In dairying the determination of alkaline phosphatase is generally used as a measure of the effectiveness of pasteurization. The stability of the enzyme is relatively high, but heat destroys it.

The alkaline phosphatase activity was determined by using sodium phenylphosphate as substrate. After an incubation of two hours (at 37 ° C) the reaction was stopped with zinc sulphate and protein material was filtered off. The phenol thus freed was measured at 610 mμ after color development with 2,6-dibromkinone-4-chloridide. The amount of the phenol liberated by the enzyme could be read directly from the phenol standards curve. Phosphatase activity is expressed as micrograms of phenol freed by the enzyme in 0.5 ml of milk during one hour.

Table 3

Cow		μg-phenol/0.5 ml milk
Aino	protein-free test feed	750
Jairu	protein-free test feed	440
Metta	protein-free test feed	395
Lila		260
Control		400

Catalase is an enzyme occurring in all animal and plant cells that can catalyze the following reaction:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The determination of catalase in milk is of practical importance. In the milk of a healthy cow there is a very small amount of catalase, whereas in colostrum and in milk from mastitic udders the enzyme occurs in large amounts. If a higher enzyme level is found in milk, it is a sign either of the presence of leucocytes which are derived from colostrum or of bacterial infection (evidence of secretion disturbances or udder disease). Milk samples from different cows show different catalase activities (JENNESS and PATTON, 1959). The composition of milk catalase is not known, but the catalases which have been isolated from animal tissues have been found to be haemproteins, and it is believed that the catalase in milk may also be of the same type of protein.

In the experiments performed by us, catalase activity was determined by an iodometric titration method (BERGMEYER, 1962). The determination of the activity of the catalase was based on the measurement of the velocity constants and extrapolation of the values to zero time to obtain the initial velocity constant k_0. For this purpose milk was allowed to react with hydrogen peroxide, which reaction was stopped with acid after set periods of time. The hydrogen peroxide which was not decomposed by the catalase was measured by adding potassium iodide and titrating the liberated iodine with thiosulphate solution, using starch as indicator. The amount of thiosulphate used depends on the amount of hydrogen peroxide (1 ml 0.05 N thiosulphate = 0.025 mMol H_2O_2).

In most cases the k_0-values vere 0 or very close to it. The maximum values obtained were 0.00090 (Metta), 0.00096 (control), 0.00101 (Aino) and 0.00087 (Jairu) sec^{-1}.

Peroxidase catalyzes the oxidation of numerous organic compounds, for example amines, phenols and leuco-color substances in the presence of hydrogen peroxide:

$$H_2O_2 + AH_2 \xrightarrow{\text{peroxidase}} 2\,H_2O + A$$

where AH_2 is the oxidizable substance or hydrogen donor. Peroxidase has been prepared in crystalline form from cow's milk (POLIS and SHMUKLER, 1953), which is one of the best peroxidase souces 'containing about 100 mg enzyme/l. When milk proteins are fractionated the peroxidase is retained in the lactalbumin fraction. Recent studies have shown evidence of lactoperoxidase heterogeneity (CARLSTRÖM, 1965).

Milk peroxidase activity fluctuates somewhat from cow to cow and day to day, but it is in general much more constant than, for instance, catalase. KIERMEIER and KAYSER (1960) have claimed that the quality of the feed has an influence on the peroxidase activity. A maize feed containing a relatively high peroxidase activity gave the milk a greater activity than did a beet feed containing a smaller activity. They observed further that the peroxidase activity was somewhat greater in summer than in winter and that there were slight breed differences.

Peroxidase activities were determined by using p-phenyldiamine as the hydrogen donor (BERGMEYER, 1962). This was oxidized in the presence of the milk peroxidase and hydrogen peroxide to the colored diimine. The increase in the color intensity (485 mμ) per unit of time is a measure of the peroxidase activity. The measurements were performed at room temperature and at pH 7.0. The results are given in Fig. 1.

Xanthine oxidase

Xanthine oxidase is an iron-molybdenum-flavoprotein which occurs also in milk. It was discovered by Schardinger in 1902 when he observed that methylene blue was decolorized by aldehydes in the presence of fresh milk. Xanthine oxidase catalyzes the following reaction:

$$\text{reduced substrate} + \text{hydrogen acceptor} \xrightarrow{\text{xanthine oxidase}} \text{oxidized substrate} + \text{reduced form of acceptor.}$$

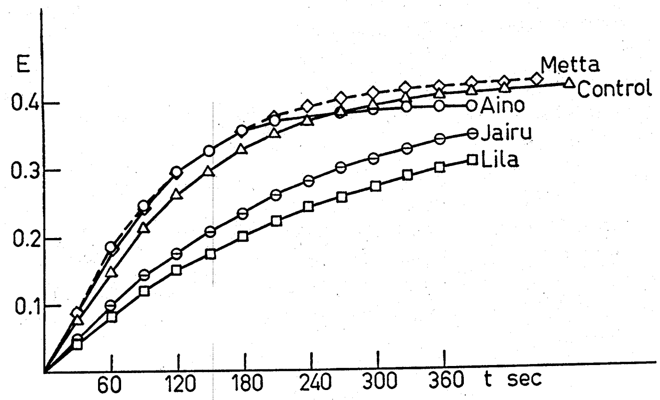

Fig. 1. Milk peroxidase activity. Relationship between extinction (E_{485} mμ) values and time of reaction (t sec.) for control and test milks.

The enzyme can oxidize many compounds, e. g. aldehydes, oxypyrines, pterines and DPN, and O_2, methylene blue, cytochrome C and 2,6 dichlorindophenol can act as electron acceptors.

Milk contains large amounts of xanthine oxidase, approximately 160 mg/l (Greenbank, 1954). The amount varies with various cows and usually increases towards the end of the lactation period, because it is associated with the fat globules and is retained in the cream after separating. Usually when concentrating or purifying this heat stable enzyme, cream or butter milk is used as the source. Crystallization of this enzyme has been performed by Avis et al. (1955). In 1954 Morton observed that the enzyme occurs in milk microsomes. The lipoprotein fragments isolated from dialyzed buttermilk contain much xanthine oxidase. A relation between the molybdenum content and xanthine oxidase of feed has also been shown to exist, a high feed molybdenum content increasing the amount of the enzyme in milk (KIERMEIER and VOGT, 1956).

Xanthine oxidase activity can be assayed in different ways, for example by determining the time of reduction of methylene blue, by measuring the oxygen consumed manometrically or by determining the reaction products. In the experiments performed in this laboratory the last-mentioned method was used (WÜTHRICH et al., 1964). Milk xanthine oxidase catalyzed the reaction:

$$\text{xanthine} + H_2O + O_2 \longrightarrow \text{uric acid} + H_2O_2$$

Xanthine was used as substrate and the reaction was carried out at pH 8.3 and 37 ° C. The reaction was followed by measuring the increase in the extinction at 292 mμ. The results in international units are given in Table 4.

Table 4

Cow		xanthine oxidase activity IU
Aino	protein-free test feed	441
Jairu	protein-free test feed	379
Lila	potato, sugar beet pulp, and urea feed	283
Control		
Liru	normal feed	320
Kelo	normal feed	473
Euru	normal feed	430
Iri	normal feed	320
Lelo	normal feed	278
Kairu	normal feed	216
		206

Side by side with the former the xanthine oxidase was determined also by using methylene blue as hydrogen acceptor and by measuring the time of reduction of the color. The results of both methods agreed closely.

Discussion and Summary

In the experiments performed on milk enzymes aldolase, amylase, alkaline phosphatase, catalase, peroxidase and xanthine oxidase were studied. The milk samples were the 0-milks (Aino, Jairu, and in some cases Metta), for the production of which the cows have been fed purified carbohydrates, urea and ammonium nitrogen being the nitrogen source, the milk of Lila which has been on a potato, sugar beet pulp and urea feed, and the control milk (the mixed milk of the cows of the Joensuu farm). The xanthine oxidase activity was further studied in the milk of a few individual cows on normal feed.

The results have been calculated, where possible, in terms of international units. The values given are the mean values of several determinations. When examining the results it can be observed that the enzymatic activities fluctuated with various cows and even on various days to some degree. All the enzymatic activities of the milk of Lila which was on the potato — sugar beet pulp feed were lower than in other milks. It is difficult to say what the explanation for this is. It cannot be due to the smaller enzymatic activity of the feed, since in the milks of cows which were given non-active purified feed the enzymatic activities were greater. The feed may in some other way have an enzymatic activity-decreasing influence. On the other hand the enzymatic activities of the milks of the test cows receiving similar synthetic feed differed remarkably from one to another. The aldolase, alkaline phosphatase and xanthine oxidase activities were greater in the milk of Aino than in that of Jairu and the control milk. Since all three above-mentioned enzymes are associated with the fat globules, it is probable that the greater enzymatic activities were influenced by the higher fat content of the milk of Aino, which was 6.0—6.4 %, while the fat content of the milk of Lila was at the same time only 3.8 %, that of Jairu 3.8—4.1 % (these Jairu values are lower than the average 4.5 % as the cow was at the end of its lactation) and the mean fat of the control milk was 4.5 %. The amylase activity which was associated with whey was the smallest in the milk of Aino. The amylase activities of the milk of Jairu and the control milk were almost the same and twice that of Aino's milk. The catalase activity was zero in most experiments or at least very close to it.

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References

AVIS, P. G., BERGEL, F., and BRAY, R. C., 1955. Cellular Constituents. The Chemistry of Xanthine Oxidase. I. The Preparation of a Crystalline Xanthine Oxidase from Cow's Milk. J. Chem. Soc., p. 1100, 1955.

BERGMEYER, H.-U., Methoden der Enzymatischen Analyse. Weinheim 1962.

CARLSTRÖM, A., The Heterogeneity of Lactoperoxidase. Acta Chem. Scand. **19,** 2387 (1965).

GRAHAM, W. R., Jr., and KAY, H. D., Phosphorus Compounds of Milk. V. The Phosphorus Partition in Milk with Preliminary Observations on Milk Phosphatase. J. Dairy Res. **5,** 54 (1933).

GREENBANK, G. R., The Xanthine Oxidase Content of Dairy Products and its Inhibition by Heat. J. Dairy Sci. **37,** 644 (1954).

GUY, E. J., and JENNESS, R., Separation, Concentration and Properties of Alpha-Amylase from Cow's Milk. J. Dairy Sci. **41,** 13 (1958).

HAAB, W., and SMITH, L. M., Variations in Alkaline Phosphatase Activity of Milk. J. Dairy Sci. **39.** 1644 (1956).

JENNESS, R., and PATTON, S., Principles of Dairy Chemistry. New York, 1959.

KIERMEIER, F., and KAYSER, C., Zur Kenntnis der Lactoperoxydase. Z. Lebensm. Untersuch. Forsch. **112,** 481 (1960).

KIERMEIER, F., and VOGT, K., Ursachen unterschiedlicher Xanthindehydrase-Aktivität in Kuhmilch aus verschiedenen Anlieferungsbezirken. Z. Lebensm. Untersuch. Forsch. **103,** 355 (1956).

MORTON, R. K., Alkaline Phosphatase in Milk. II. Purification of the Enzyme. Biochem. J. **55,** 795 (1953).

MORTON, R. K., The Lipoprotein Particles in Cow's Milk. Biochem. J. **57,** 231 (1954).

POLIS, B. D., and SHMUKLER, H. W., Aldolase in Bovine Milk. J. Dairy Sci. **33,** 619, 1950.

POLIS, B. D., and SHMUKLER, H. W., Crystalline Lactoperoxidase. I. Isolation by Displacement Chromatography. J. Biol. Chem. **201,** 475 (1953).

RICHARDSON, G. A., and HANKINSON, C. L., Amylase in Cow's Milk. J. Dairy Sci. **19,** 761 (1936).

RICHTERICH, R., and COLOMBO, J. P., Ultramikro-Methoden im Klinischen Laboratorium. Ärztl. Lab. **8,** 33 (1962).

SCHLOEMER, A., SCHLOEMER, R., and BLEYER, B., Die Milchamylase, ihr Vorkommen, ihre Eigenschaften und ihre Anwendung zum Nachweis der Pasteurisierung. Milchw. Forsch. **20,** 59 (1939).

SHAHANI, K. M., Milk Enzymes: Their Role and Significance. J. Dairy Sci. **49,** 907 (1966).

SYVÄOJA, E.-L., and VIRTANEN, A. I., Fractionation of Milk Proteins, Especially of Milk Produced on Protein-free Feed with Urea and Ammonium Salt as the Sole Source of Nitrogen. Enzymologia **29,** 205 (1965).

VIRTANEN, A. I., and SYVÄOJA, E.-L., Proteins of Cow's Milk Produced on Normal Feed and Feed Containing Urea and Ammonium Salts as the Sole Nitrogen Sources. Suomen Kemistilehti B. **37,** 134 (1964).

VIRTANEN, A. I., Milk Production of Cows on Protein-Free Feed. Science **30,** 1603 (1966).

WÜTHRICH, S., RICHTERICH, R., and HOSTETTLER, H., Untersuchung über Milchenzyme. I. Enzyme in Kuhmilch und Frauenmilch. Z. Lebensm. Unters. Forsch. **124,** 336 (1964).

Zusammenfassung

SYVÄOJA, E.-L., und VIRTANEN, A. I.: **Untersuchungen über Enzyme in Milch von Kühen, die normal und proteinfrei gefüttert wurden.** *„Milchwissenschaft"* **23.** *(4) 200—204 (1968).*

24 Enzymaktivität (eiweißfreie Fütterung).

Auf der Grundlage von Vorversuchen kann bereits gesagt werden, daß synthetisches Futter, wobei Harnstoff und Ammoniumsalz die einzigen Stickstoffquellen sind, keinen Einfluß auf die normale enzymatische Aktivität der Milch hat. Darüberhinaus widerlegen die bisher erhaltenen Ergebnisse die Meinungen anderer Autoren, daß durch eine geringere enzymatische Aktivität des Futters die enzymatische Aktivität der Milch reduziert wird. Es ist wahrscheinlicher, daß diese Differenzen auf Unterschiede in der Laktationsperiode, auf Schwankungen im Fettgehalt und andere individuelle Unterschiede zurückzuführen sind.

Dok.-Ref.

SYVÄOJA, E.-L., and VIRTANEN, A. I.: **Studies of enzymes in milk produced with normal feed and protein-free feed.** *„Milchwissenschaft"* **23.** *(4) 200—204 (1968).*

24 Enzyme activity (protein-free feeding).

On the basis of the preliminary experiments it can already be observed that the synthetic feed — urea and ammonium salts being the sole nitrogen source — does

not have any influence on the normal enzymatic activities of milk. In addition to this the results refute the claims of other authors that the smaller enzymatic activity of the feed would have an enzymatic activity — decreasing influence. It is more probable that the differences are due to the lactation period, fat content and individual differences.

SYVÄOJA, E.-L., et VIRTANEN, A. I.: **Etudes sur les enzymes dans le lait de vaches alimentées avec du fourrage exempt de protéines.** *„Milchwissenschaft“* **23.** *(4) 200—204 (1968).*

24 Activité enzymatique (fourrage exempt de protéines).

SYVÄOJA, E.-L., y VIRTANEN, A. I.: **Estudios sobre las encimas en la leche de vacas cuya alimentación carecía de proteínas.** *„Milchwissenschaft“* **23.** *(4) 200—204 (1968).*

24 Actividad encimática (alimentación de vacas que carece de proteinas).